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Successful passive and active immunization of cynomolgus monkeys against hepatitis E

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ABSTRACT **Virtually full protection against hepatitis E and partial or complete protection against infection with hepatitis E virus (HEV) were achieved in passively or actively immunized cynomolgus monkeys. Hepatitis, viremia, and shedding of the virus in feces were detected in all nonimmunized animals that were challenged with HEV. HEV titers detected by reverse transcriptase PCR were higher in feces than in serum of nonimmunized animals. Anti-HEV antibody titers at the time of challenge ranged between 1:40 and 1:200 in animals passively immunized with convalescent plasma from a cynomolgus monkey previously infected with HEV and between 1:100 and 1:10,000 in animals actively immunized with a recombinant 55-kDa open reading frame 2 protein. The estimated 50% protective titer of passively acquired anti-HEV antibodies was 1:40. Although only one of four passively immunized animals showed histopathologic evidence of hepatitis, all four were infected after challenge; however, the titers of HEV in serum and feces were lower in the passively immunized animals than in the nonimmunized group. The actively immunized animals developed neither hepatitis nor viremia when challenged with HEV and virus was either not detected or was present in low titer in feces. The protective response was a function of the ELISA anti-HEV antibody titer at the time of challenge and the immunization schedule.**

The hepatitis E virus (HEV) is a small nonenveloped RNA virus that causes one type of acute, self-limiting hepatitis (hepatitis E) in developing countries of Asia and Africa (1). In the New World, the virus has been detected in Mexico (2), but only limited surveillance has been carried out in this region and it is suspected that hepatitis E exists elsewhere in the hemisphere. The disease caused by HEV was first recognized in 1980 (3, 4) and the virus was first visualized in 1983 (5). The virus causes both epidemic and endemic disease. The former is usually associated with massive fecal contamination of water and the latter is associated with inadequate personal and public hygiene. The epidemiology of HEV is similar to that of another fecally transmitted hepatitis virus, hepatitis A virus (HAV), but HAV is more readily transmitted, causes more infections, and has a wider distribution worldwide. Nevertheless, HEV causes more clinical disease than HAV in developing countries (6) because most HAV infections occur in children and are associated with little or no disease, whereas HEV infections occur in adults and are associated with hepatitis (7). Furthermore, a high rate of mortality has been reported in pregnant women with hepatitis E in these populations (8).

A vaccine against HEV might prevent epidemics and sporadic cases of hepatitis E in developing countries and could provide protection to travellers to those regions. Recently, it was shown that individuals with naturally acquired antibodies to HEV were protected against hepatitis E during an epidemic in Pakistan (9), and preliminary evidence suggested that anti-HEV antibodies raised against a recombinant HEV fusion protein protected cynomolgus monkeys against challenge with HEV (10). However, thorough evaluation of the efficacy of experimental vaccination could be performed only after certain requirements were fulfilled. First, sensitive serological tests for anti-HEV in a particular animal model had to be developed (11, 12). Second, a well characterized viral stock of known infectivity titer in the animal model was needed (13). Third, appropriate materials for immunization had to be obtained. In the present study, titered convalescent plasma from an experimentally infected cynomolgus monkey was used for passive immunization, and a putative structural HEV protein expressed in insect cells (11, 12) was used for active immunization of cynomolgus monkeys prior to challenge with 1000–10,000 cynomolgus 50% infectious doses (CID_{50}) of the SAR-55 strain of HEV.

MATERIALS AND METHODS

Primates. Eighteen cynomolgus monkeys (*Macaca fascicularis*) that were anti-HEV antibody negative (<1:10) in a sensitive ELISA (11, 12) were used in this study. Primates were individually housed under BL-2 biohazard containment. The housing, maintenance, and care of the animals met or exceeded all requirements for primate husbandry. Three monkeys died during the course of the experiment. These are identified in *Results*.

HEV Challenge Stock. A suspension (in fetal bovine serum) of feces containing the Pakistani HEV strain SAR-55, diluted to contain 10,000 or 1000 CID_{50} (12), was used for intravenous inoculation of animals.

Inocula for Passive Immunization. One of the eighteen monkeys, cyno-384, was infected with 0.5 ml of a 10% pooled stool suspension containing two Chinese HEV isolates, KS1-1987 and KS2-1987 (13). Late convalescent plasma from cyno-384 with an anti-HEV antibody titer of 1:10,000 was collected and infused into two pairs of cynomolgus monkeys.

Inocula for Active Immunization. Baculovirus recombinant-expressed 55-kDa open reading frame 2 protein (11, 12) was purified from 5×10^8 Sf9 cells harvested 7 days postinoculation. The infected cells were centrifuged, resuspended in 10 ml of 10 mM Tris·HCl, pH 8.0/50 mM NaCl, containing 40 μg of phenylmethylsulfonyl fluoride per ml (Sigma), and sonicated to disrupt the cells; the lysate was centrifuged at

Abbreviations: HEV, hepatitis E virus; HAV, hepatitis A virus; CID_{50}, cynomolgus 50% infectious dose; ALT, alanine aminotransferase.

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90,000 × g at 4°C for 30 min. The supernatant was loaded onto a DEAE-Sepharose CL-6B (Pharmacia) column equilibrated with 10 mM Tris·HCl, pH 8.0/50 mM NaCl. The column was washed with loading buffer and the 55-kDa protein was eluted in 10 mM Tris·HCl, pH 8.0/250 mM NaCl. Fractions containing the 55-kDa protein were combined and the protein was precipitated by addition of 3 g of $(NH_4)_2SO_4$ to 10 ml of the protein solution. The protein pellet was dissolved in 10 mM Tris·HCl, pH 8.0/50 mM NaCl.

Three milligrams of the purified 55-kDa protein was precipitated with alum. The efficiency of precipitation was 99.7%, as determined by ELISA of the residual soluble antigen. The protein–alum complex was stored at +4°C for up to 3 months.

Inoculation Schedule. Approximately 1% of the blood of cyno-396 and cyno-399 and 10% of the blood of cyno-401 and cyno-402 was replaced with anti-HEV plasma from cyno-384. Animals were challenged with 1000 CID_{50} of HEV 2 days after infusion of the plasma.

Eight cynomolgus monkeys were immunized by intramuscular injection with 0.5 ml of vaccine containing 50 μg of the alum-precipitated 55-kDa protein. Four monkeys received a single dose and four monkeys received two doses separated by 4 weeks. Primates were challenged intravenously with 1000–10,000 CID_{50} of HEV 4 weeks after the last immunization.

Five cynomolgus monkeys served as controls. Ten percent of the blood of cyno-405 was replaced with anti-HEV-negative plasma obtained from cyno-384 prior to infection with HEV. Cyno-412 and -413 received one dose of placebo (0.5 ml of phosphate-buffered saline) and cyno-397 and -849 received two doses of placebo. The control animals were challenged with 1000–10,000 CID_{50} of HEV.

Monitoring of Primates. Percutaneous needle biopsies of the liver and samples of serum and feces were collected prior to inoculation and weekly for 15 weeks after inoculation. Sera were assayed for levels of alanine aminotransferase (ALT) with commercially available tests (Metpath, Rockville, MD). Biochemical evidence of hepatitis was defined as a 2-fold or greater increase in ALT. Liver biopsies were examined under code as described (14). The anti-HEV ELISA has been described (11, 12). RNA extraction and reverse transcriptase (RT)-PCR were performed as described (14) except that RNA from 100 μl of serum or from 100 μl of 10% fecal suspension was extracted with TriZOL reagent (GIBCO/BRL) according to the manufacturer's protocol. For quantification, PCR-positive serial sera or feces from each animal were combined and serially diluted in 10-fold increments in calf serum. One hundred microliters of each dilution was used for RNA extraction and RT-PCR. The PCR protocol used in this study could detect as few as 10 CID_{50} of HEV per ml of serum and as few as 100 CID_{50} per g of feces.

Statistical Analysis. Peak ALT values of weekly serum samples for 5 weeks prior to inoculation and for 15 weeks postinoculation were expressed as ratios (post-/pre-) for each animal. The geometric mean of the ratios from the control group of animals was compared with that from the passively or actively immunized animals by the Simes test (15).

The durations of viremia and virus shedding in feces and the HEV genome titers in the control group of animals were compared with those in passively or actively immunized animals by the Wilcoxon test (16). The same test was used to compare the above parameters between passively and actively immunized animals.

For statistical analysis, serum samples that had <10 HEV genomes in 1 ml of serum were assigned a titer of 1:1 and fecal samples that had <100 HEV genomes in 1 g of feces were assigned a titer of 1:10.

RESULTS

Course of Hepatitis E Infection in Nonimmunized Animals. In three of five nonimmunized animals that were challenged with HEV, biochemical evidence of hepatitis was documented by at least a 2-fold increase in serum ALT values. In two animals, significant increases in ALT activity were not found. However, histopathological data documented hepatitis in all five animals (Table 1). Necroinflammatory changes ranged between 1+ and 2+ on a scale of 1+ to 4+ and were temporally associated with elevations of ALT activities in those animals with such elevations.

All control animals seroconverted to HEV 3–5 weeks postchallenge (Table 2) and developed maximum anti-HEV antibody titers ranging from 1:1000 to 1:32,000. There was a good correlation between the severity of infection, hepatitis, and the level of anti-HEV response. Cyno-405, which had the highest cumulative score for hepatitis, also had the longest period of viremia and viral excretion and the highest level of anti-HEV antibody (Table 1). The duration of viral shedding in feces was the same as, or longer than, that of the viremia. For all of the control animals, titers of the HEV genome in serum were lower (10^{-3}–$10^{-4.7}$) than the titers in feces ($10^{-5.7}$–10^{-7}). In all five of these animals, viremia and virus shedding in feces were detected for 4–11 weeks and for an average of 4.2 weeks after seroconversion (range, 2–9 weeks).

Passive Immunization. Cyno-396 and -399, which had ≈1% of their blood replaced with anti-HEV-positive convalescent plasma, had an anti-HEV antibody titer of 1:40 when it was determined 2 days posttransfusion (at the time of challenge) (Table 1). A 2-fold fall in anti-HEV antibody titer was observed in both animals 1 week posttransfusion and anti-HEV antibodies fell below the detectable level (<1:10) by 2 weeks posttransfusion. Anti-HEV antibody was again detected 5 weeks postchallenge in cyno-396 and 4 weeks postchallenge in cyno-399, indicating that infection with HEV had occurred. The maximum anti-HEV antibody titer (1:8000) was reached 9–10 weeks postchallenge. Neither cynomolgus monkey demonstrated a significant elevation of ALT activity after challenge. However, histologic evidence of hepatitis was detected in cyno-396 and the HEV genome was detected in serum and feces from both animals (Table 1).

Cyno-401 and -402 had ≈10% of their blood replaced with convalescent plasma. Two days posttransfusion, at the time of challenge, the anti-HEV antibody titer in both cynomolgus monkeys was 1:200 (Table 2). Anti-HEV antibody was detected continuously in both animals during the 15 weeks after challenge and reached a maximum titer of 1:4000 in cyno-401 but only 1:80 in cyno-402. Biochemical and histologic analyses did not reveal hepatitis in either animal. However, in both animals, HEV viremia and fecal shedding of virus were observed, indicating that infection had occurred (Table 1). Thus, passive immunoprophylaxis that achieved a higher titer of antibody protected cynomolgus monkeys against hepatitis after challenge with HEV.

Active Immunization. Four primates immunized with one 50-μg dose of the 55-kDa protein developed antibody to the recombinant protein ranging in titer from 1:100 to 1:10,000 (Table 2). One (cyno-013) died of an anesthesia accident 9 weeks after challenge and is included in the analyses (Table 1). The four animals that received two doses of the antigen developed HEV antibodies with titers of 1:10,000. Two of the four monkeys died after intravenous challenge with HEV. This may have also been the result of an anesthesia accident but the exact etiology could not be determined. These two monkeys were deleted from further analyses. None of the 6 remaining animals developed abnormal ALT levels or histologic evidence of hepatitis after challenge (Table 1). Cynomolgus monkeys immunized with either one or two doses of

Table 1. Histopathological, biochemical, serological, and virological profiles of vaccinated and control animals challenged with HEV

Animal and category	Anti-HEV-positive plasma (%) or 55-kDa protein (μg)	Cumulative score of histopathology (no. of weeks detected)*	Peak ALT value units/liter (week)		Anti-HEV antibody titer at time of challenge	HEV genome			
						Serum		Feces	
			Pre-inoculaton	Post-inoculation		Week detected (duration)	Mean log_{10} titer per ml	Week detected (duration)	Mean log_{10} titer per g
Control									
Cyno-405	0	10+ (8)	67 (0)	143 (9)	<1:10	1–11 (11)	3	1–11 (11)	5.7
Cyno-412	0	2+ (1)	34 (0)	45 (3)	<1:10	1–4 (4)	3	2–5 (4)	7
Cyno-413	0	4+ (4)	44 (0)	261 (6)	<1:10	2–7 (6)	4.7	1–7 (7)	7
Cyno-849	0	1+ (1)	79 (2)	133 (2)	<1:10	1–4 (4)	3.7	1–4 (4)	7
Cyno-397	0	3+ (3)	52 (3)	139 (7)	<1:10	2–6 (5)	4.7	1–7 (7)	7
Passive IP†									
Cyno-396	1%	1+ (1)‡	33 (0)	53 (6)	1:40	3–5 (3)	4	1–6 (6)	5.7
Cyno-399	1%	0 (0)	69 (0)	63 (11)	1:40	2–4 (3)	3	1–4 (4)	4
Cyno-401	10%	0 (0)	55 (0)	45 (5)	1:200	3 (1)	3.6	1–3 (3)	5.7
Cyno-402	10%	0 (0)	59 (0)	35 (2)	1:200	4–6 (3)	1	2–6 (5)	5.7
Active IP†									
Cyno-003	50 μg	0 (0)	34 (3)	50 (6)	1:10,000	0	<1	2–4 (3)	3
Cyno-009	50 μg	0 (0)	34 (2)	38 (6)	1:1000	0	<1	0	<2
Cyno-013§	50 μg	0 (0)	44 (3)	36 (7)	1:100	0	<1	1–2 (2)	3
Cyno-414	50 μg	0 (0)	65 (0)	73 (8)	1:1000	0	<1	2 (1)	2
Cyno-398	50 × 2 μg	0 (0)	31 (0)	41 (2)	1:10,000	0	<1	0	<2
Cyno-407	50 × 2 μg	0 (0)	150 (0)	213 (4)	1:10,000	0	<1	0	<2

*Necroinflammatory changes in the liver were rated as 1+, 2+, 3+, and 4+, and the weekly scores were summed.
†Immunoprophylaxis.
‡Necroinflammatory changes rated 1+ were detected during 2 weeks in cyno-396; however, they were consistent with viral hepatitis during only 1 week.
§Cyno-013 died 9 weeks after challenge.

the 55-kDa protein did not develop viremia. However, three of four animals that received one dose of the immunogen excreted virus in their feces. In contrast, virus shedding was not observed in either of the two challenged animals that had received two doses of the vaccine.

Most of the actively immunized animals developed higher anti-HEV antibody titers than did passively immunized animals. However, cyno-013 had an anti-HEV antibody titer of 1:100 at the time of challenge, compared with a titer of 1:200 in two animals immunized passively with anti-HEV plasma. Cyno-013, however, demonstrated greater protection against HEV infection than the passively immunized animals. Cyno-009, which had an anti-HEV antibody titer of 1:1000 at the time of challenge, was completely protected against hepatitis and HEV infection (Table 1). In contrast, cyno-003 was infected and shed HEV in feces, even though it had an anti-HEV antibody titer of 1:10,000 at the time of challenge. However, neither hepatitis nor viremia was detected in this animal or in other cynomolgus monkeys that received one dose of immunogen and had HEV antibody titers of 1:10,000 or greater.

Comparison of Course of HEV Infection in Control and Immunized Animals. As measured by histopathology, all immunized animals, with the exception of one of the passively immunized monkeys, were protected against hepatitis after intravenous challenge with HEV. Comparison of mean values for severity of hepatitis and level of viral replication between the control group and the passively and actively immunized animals indicated that, in general, the severity of infection was inversely related to the anti-HEV antibody titer at the time of challenge and diminished in the order unimmunized > passive immunization (1%) > passive immunization (10%) > active immunization (one dose) > active immunization (two doses) (Tables 1 and 3). However, the number of animals in each of the two subgroups of passively and actively immunized animals was not sufficient to permit statistical analysis. Therefore, statistical analysis was performed for combined passively immunized and combined actively immunized groups in comparison with the combined control groups (Table 3).

The histopathology scores and duration of histologic changes in the control group were statistically different from those of passively or actively immunized animals (Table 3). The higher post-/preinoculation ratios of peak ALT values in the control group were statistically significant when compared with those of the passively or actively immunized animals, indicating protection against biochemical manifestations of hepatitis in both groups of immunized animals. The

Table 2. Anti-HEV antibody profiles in control and immunized cynomolgus monkeys

Control animal	Anti-HEV antibody		Passively immunized animal	Anti-HEV antibody		Actively immunized animal	Anti-HEV antibody		
	Titer (week first detected)	Maximum titer (week)		Titer at time of challenge	Maximum titer (week after challenge)		Maximum titer (week after 1st immunization)	Maximum titer (week after 2nd immunization)	Maximum titer (week after challenge)
Cyno-405	1:80 (3)	1:32,000 (9)	Cyno-396	1:40	1:8000 (10)	Cyno-003	1:10,000 (3)		1:10,000 (5)
Cyno-412	1:100 (5)	1:10,000 (7)	Cyno-399	1:40	1:8000 (9)	Cyno-009	1:10,000 (3)		1:10,000 (1)
Cyno-413	1:100 (5)	1:10,000 (7)	Cyno-401	1:200	1:4000 (6)	Cyno-013	1:100 (2)		1:10,000 (3)
Cyno-849	1:100 (3)	1:1000 (5)	Cyno-402	1:200	1:80 (12)	Cyno-414	1:1000 (3)		1:1000 (0)
Cyno-397	1:100 (3)	1:10,000 (7)				Cyno-398	1:1000 (3)	1:10,000 (5)	1:10,000 (0)
						Cyno-407	1:1000 (4)	1:10,000 (5)	1:10,000 (0)

Table 3. Summary of mean values of HEV infection in control and immunized animals

Category of animals (*n*)	Histopathology: Mean of cumulative score	Histopathology: Weeks	GM* of peak ALT, units/liter: Pre-inoculation	GM* of peak ALT, units/liter: Post-inoculation	GM* of peak ALT, units/liter: Ratio	Anti-HEV antibody titer at time of challenge	HEV genome: Serum: Mean no. of weeks	HEV genome: Serum: Mean $\log_{10}$ titer per ml	HEV genome: Feces: Mean no. of weeks	HEV genome: Feces: Mean $\log_{10}$ titer per g
Control (5)	4+	3.4	53	125	2.4	<1:10	6	3.8	6.6	6.7
Passive 1% (2)†	0.5+	0.5	48	58	1.2	1:40	3	3.5	5	4.9
Passive 10% (2)†	0	0	57	40	0.7	1:200	2	2.3	4	5.7
Active 1 dose (4)†	0	0	43	47	1.1	1:3,025	0	<1	1.5	2
Active 2 doses (4)†	0	0	68	93	1.4	1:10,000	0	<1	0	<2

α, $P < 0.01$; β, $P < 0.05$; γ, not significant.
*Geometric mean.
†Passive and active immunoprophylaxis.

duration of viremia and the titer of HEV in the feces were significantly lower in both groups of immunized animals than in the control group. Differences in the duration of virus shedding and titer of HEV in the serum, however, were not statistically different between the control group and the passively immunized group, although these parameters were significantly different when the control group was compared with the actively immunized group. Significant differences were also found between passively and actively immunized groups of animals for duration of viremia and fecal shedding as well as for HEV titers.

DISCUSSION

In this study, we found that both passively and actively acquired anti-HEV antibodies protected cynomolgus monkeys against hepatitis after challenge with virulent HEV. Although all five nonimmunized cynomolgus monkeys developed histologic evidence of hepatitis when challenged with 1000–10,000 CID_{50} of SAR-55, both animals with passively acquired antibody titers of 1:200 were protected from hepatitis and one of two animals with an antibody titer as low as 1:40 also did not develop hepatitis. However, passively immunized animals that did not manifest biochemical or histologic signs of hepatitis still were infected. Passive immunization with HEV antibody titers as high as 1:200 was not able to protect the animals against HEV infection since viral genomes were detected in serum and feces after challenge, although at lower titer than in nonimmunized animals.

The anti-HEV-positive plasma used in this study for passive immunoprophylaxis was collected during early convalescence from cyno-384, and it provided a relatively high HEV antibody titer of 1:10,000. Nevertheless, it was not possible to prevent infection with HEV in monkeys infused with this plasma. Titers of anti-HEV antibodies in human populations where hepatitis E is endemic have been lower than this; geometric mean titers of anti-HEV antibodies determined by the same anti-HEV ELISA as used in this study fell from >1:5000 during an epidemic in Pakistan to <1:500 20 months later (9) and the anti-HEV titers of individual positive sera in a normal population in India were all <1:1000 (7). Therefore, pooled human immunoglobulin collected in regions where HEV is endemic is unlikely to have sufficient anti-HEV antibodies to be useful for passive immunization. Indeed, protection against hepatitis E was not found when such immunization was attempted (2, 17, 18). To be effective passive immunoprophylaxis for hepatitis E will likely require careful selection of plasma units with a high titer of anti-HEV antibody or active immunization of plasma donors with a vaccine similar to the one described here.

In contrast, actively immunized animals demonstrated complete protection against hepatitis and more effective resistance to HEV infection than did passively immunized animals. This difference between the two types of immunization is unlikely to be due to immunological differences between the Chinese strains of HEV (13) used to produce the anti-HEV plasma and the Pakistani strain of HEV (11) used as the source of the recombinant immunogen and the challenge pool, because no differences were found in amino acid sequences between the capsid protein of the Chinese KS-1 and KS-2 strains and that of the SAR-55 strain (13). In contrast to results obtained from the passively immunized animals, viremia was not detected in actively immunized animals after challenge with HEV. An anti-HEV antibody titer as high as 1:10,000 could be achieved in cynomolgus monkeys after one or two immunizations with the recombinant 55-kDa protein. Although one monkey (cyno-013) developed a titer of 1:100 after active immunization, this level still prevented hepatitis and viremia.

A single dose of vaccine prevented HEV viremia, but viral shedding in feces was still detected. This suggests that a single dose of vaccine administered, for example, to individuals before foreign travel would protect them from hepatitis E in high-risk environments. However, two doses of vaccine prevented all signs of hepatitis and HEV infection was completely prevented. Since active immunization induced anti-HEV antibody levels as high as 1:10,000, even with a single dose of vaccine, and demonstrated better protection against both hepatitis and HEV infection than did passive immunization, it is likely that vaccination will be more useful for prophylaxis against hepatitis E than the use of immune globulin.

These results are very similar to results we reported previously (19) for passive and active immunoprophylaxis of nonhuman primates against hepatitis A; passive immunoprophylaxis prevented hepatitis but not infection, whereas vaccination prevented not only hepatitis but infection with HAV as well. It is interesting that this study of immunoprophylaxis for HEV parallels the previous study of immunoprophylaxis against HAV, both in determination of the titer of antibody that protected (<1:100) and in outcome after intravenous challenge with virulent virus. Other studies have demonstrated efficacy of comparable titers of passively and actively acquired anti-HAV antibody in humans and have confirmed the predictive value of studies of primates in hepatitis research (20, 21). It is therefore highly likely that our results in cynomolgus monkeys will be predictive of protection in humans.

In contrast to a previous preliminary report of vaccination of cynomolgus monkeys against hepatitis E with a recombi-

nant protein of HEV expressed in *Escherichia coli* (10), the candidate vaccine used in the present study partially or completely protected after only one or two 50-μg doses of alum-adjuvanted vaccine. In the previous study, a comparably adjuvanted vaccine failed to afford any protection after two 80-μg doses and a third dose of aqueous vaccine was required to protect a single vaccinated monkey (10). Thus, the vaccine and vaccination schedules documented here appear to provide a practical approach to vaccination of at-risk populations against hepatitis E.

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